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# Occurrence of Pyruvic Acid in Capsular Polysaccharides from Various *Klebsiella* Species

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Capsular polysaccharide materials from several different klebsiella serotypes were demonstrated to contain an α-keto acid characterized as pyruvic acid. Linkage to the capsular polysaccharides was shown to be acid labile and alkali stable, suggesting ketosidic rather than ester linkages.

Alpha keto acids, shown in most instances to be pyruvic acid, are known to be present in polysaccharides of several *Xanthomonas* species (5, 8, 12, 14, 15), three *Rhizobium* species (3, 7, 8), *Corynebacterium insidiosum* (6), a pseudomonas (5, 12), various pneumococci (3, 7), *Klebsiella rhinoscleromatis* 13884, and several other klebsiellae (7, 11, 18). Pyruvic acid also occurs in polysaccharides of the red seaweed agar-agar (9) and was recently found in colanic acid, on exopolysaccharide produced by the enterobacteria (10, 17).

This work documents the characterization of pyruvic acid found in capsular polysaccharide preparations of several strains of *K. rhinoscleromatis* and klebsiella serotypes, A(1), B(2), C(3), D(4), E(5), F(6), and 68, and thereby confirms our preliminary report (11). Evidence in support of a ketosidic linkage between the pyruvic acid containing aqueous soluble polysaccharides extracted from these klebsiellae is also recorded.

## MATERIALS AND METHODS

**Bacterial strains.** Klebsiella types A(1), B(2), C(3), D(4), E(5), F(6), and 68 were obtained from W. H. Ewing, Center for Disease Control, Atlanta, Ga. Strains G-SS-8, G-SS-11, and G-SS-12 were isolated from cases of rhinoscleromatis by G. Godoy, Departamento Microbiologia, Facultad de Medicina, Universidad de El Salvador, San Salvador, Central America. Other *K. rhinoscleromatis* strains, 6908, 13884, and C-5046, were obtained from the American Type Culture Collection, Rockville, Md.

**Growth conditions.** Organisms were grown with aeration for 36 hr at 37 C in a liquid medium containing glucose, salts, and yeast extract dialysate as previously reported (18).

**Preparation of capsular polysaccharides.** Cells were killed by the addition of 10 volumes of 95% ethanol after adjusting the culture to 0.1 M with sodium acetate. The precipitated cells and polysaccharides were harvested and converted to an acetone powder. Capsular polysaccharides were then extracted into deionized water by heating 5% suspensions at 60 C for 2 hr with occasional shaking. Bacterial debris was removed by centrifugation at 13,000 × *g* for 20 min, supernatant solutions were adjusted to 0.1 M with sodium acetate, and polysaccharides were again precipitated with 10 volumes of 95% ethanol. The ethanol precipitates were acetone powdered, redissolved in water, dialyzed against daily changes of deionized water for 96 hr before lyophilization, and stored in vacuo over $P_2O_5$. All preparations yielded precipitin bands in double immunodiffusion assays done on microscope slides (13), using homologous type specific klebsiella antisera purchased from Difco.

**Treatment of dialysis tubing.** Dialysis tubing, 27 mm viscasing, treated by repeated heating in aqueous 1% sodium carbonate and 0.01 M ethylenediaminetetraacetic acid followed by washing with deionized water to remove heavy metals, contained no detectable sulfur (xanthates), extractable soluble materials containing keto acids, uronic acids, or reducing sugars.

**Alpha keto acids.** Sodium pyruvate was obtained from Eastman Co., Rochester, N.Y. Lithium β-hydroxypyruvate was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Sodium α-keto butyrate and sodium glyoxylate were purchased from Sigma Chemical Co., St. Louis, Mo. A sample of ammonium β-mercaptopyruvate was generously provided by Nicholas Kredich, Department of Medicine, Duke University Medical Center, Durham, N.C.

**Analytical methods.** Pyruvic acid was quantified by conversion to the 2,4-dinitrophenylhydrazone derivative and assay according to the toluene extraction method of Friedemann and Haugen (4), and also by enzymatic assay, using crystalline lactic dehydrogenase [I.U.B. number 1.1.1.27; Worthington Biochemical Corp., Freehold, N.J. (2)]. Thin-layer chromatography of ether extracts of acid hydrolysates was carried out on cellulose plates. Solvent systems used were (A) ethyl-acetate-pyridine-acetic acid-water (5:5:1:3) and (B) *n*-butanol-pyridine-water (6:4:3).

**Catalytic hydrogenation.** Ethanol (95%) solutions of

the α-keto acid 2,4-dinitrophenylhydrazone derivatives were catalytically reduced in a low-pressure hydrogenation apparatus, utilizing a trace of 10% palladium on charcoal at an initial pressure of 4 atm for 24 hr (1).

**Amino acid chromatography.** Amino acid analyses were done on a modified Beckman 120-C amino acid analyzer system previously described (16), using amino acid standards obtained from the Beckman Co., Palo Alto, Calif. L-α-Amino-n-butyric acid was purchased from Mann Research Laboratories, New York, N.Y.

## RESULTS

The presence of α-keto acids in acid hydrolysates of the various polysaccharide preparations was indicated by positive results in the Friedemann and Haugen procedure after conversion to the 2,4-dinitrophenylhydrazone derivatives (4). Ether extraction of the acid hydrolysates yielded a compound which exhibited both carbonyl group reactivity and chromatographic mobility identical to pyruvic acid in all strains examined (Table 1). Because salt and free acid forms of the unknown and standard reference keto acid materials yielded different mobilities on chromatograms, especially in acidic solvent systems, standards were converted to free acid form by treatment with Dowex 50 ($H^+$) before comparing with the salt-free ether-extracted materials.

The α-keto acid from each klebsiella polysaccharide was reactive with lactic dehydrogenase at rates comparable to known pyruvic acid, ruling out several α-keto acids with the exception of pyruvic, glyoxylic, and β-hydroxypyruvic acids (2). The latter two possibilities were excluded by thin-layer chromatography (Table 1).

Graded acid hydrolysis as a function of time revealed that 80% of the total pyruvic acid could be liberated in 0.01 N HCl at 100 C for 30 min. Complete release of the keto acid was obtained at 100 C in 1.0 N HCl in 30 min, by 0.1 N HCl in 1 hr, and by 0.01 N HCl in 1.5 to 2 hr. However, standing in 0.2 N NaOH at room temperature for a period up to 24 hr resulted in no loss of acid-hydrolyzable pyruvate from the polysaccharide. This was shown by subsequent hydrolysis in 1 N HCl at 100 C for 1.5 hr, which yielded quantitative recovery of the pyruvic acid from the alkali-treated preparations (Table 2). Volatility of the pyruvic acid required the use of sealed tubes or Teflon-lined screw-cap tubes for hydrolysis.

Characterization of the α-keto acid in the various klebsiella polysaccharides was achieved by hydrolyzing, in 0.01 N HCl for 90 min at 100 C, a mixture containing approximately 2 mg of capsular polysaccharides from strains A(1), B(2), C(3), D(4), E(5), F(6), 68, and G-SS-12, followed by reaction with excess 2,4-dinitrophenylhydrazine. At this point, Friedemann and Haugen assay indicated that 7.9 μmoles of pyruvic acid was present. This agreed within 5% of the amount expected, based on the pyruvate contents previously determined for each individual sample. The resulting hydrazone was extracted into diethylether; the solvent was evaporated, and the bulk of the sample was subjected to catalytic hydrogenation (1). Quantitative assay of a sample of the material extracted with diethylether indicated 6.4 μmoles of pyruvate phenylhydrazone in the ether extract. Dark-colored side products of the 2,4-dinitrophenylhydrazine-phenylhydrazone reduction mixture were separated from possible amino acids by passage through a column of Dowex-50 ($H^+$), followed by elution of any amino acids with 10 volumes of 4 N HCl. HCl was removed from the eluate by repeated water addition and evaporation to dry-

TABLE 1. *Chromatographic mobilities of the ether-extractable α-keto acid from klebsiella polysaccharides*

| Compound | Solvent system[a] | |
|---|---|---|
| | $R_{pyruvic\ acid}$ | $R_{pyruvic\ acid}$ |
| Sodium glyoxylate | 0.32 | ND |
| Glyoxylic acid | ND[b] | 0.15 |
| Sodium pyruvate | 1.00 | 0.75 |
| Pyruvic acid | ND | 1.00 |
| Lithium β-hydroxypyruvate | 0.59 | ND |
| β-Hydroxypyruvic acid | ND | 0.39 |
| Ammonium β-mercaptopyruvate | 0.42 | ND |
| β-Mercaptopyruvic acid | ND | 0.26 |
| Sodium α-ketobutyrate | 1.15 | 1.12 |
| α-Ketobutyric acid | ND | 1.18 |
| Klebsiella type or strain | | |
| A(1) | 1.00 | 1.09 |
| B(2) | | |
| C(3) | 0.99 | 1.09 |
| D(4) | 0.99 | 1.06 |
| E(5) | 0.99 | ND |
| F(6) | 1.00 | 1.09 |
| 68 | 1.00 | 1.03 |
| C-5046 | 1.00 | ND |
| 6908 | 0.99 | 0.91 |
| G-SS-8 | 0.99 | 0.97 |
| G-SS-11 | 1.00 | 1.00 |
| G-SS-12 | 1.00 | 1.09 |
| 13884 (preparation no. 1) | 1.00 | 1.02 |
| 13884 (preparation no. 2) | 1.00 | 1.08 |

[a] Spots were visualized after chromatography by spraying with 0.5% *p*-nitrophenylhydrazine followed by 1 N NaOH. The free acid forms of the standard reference compounds were prepared from the salts by treatment with Dowex 50 ($H^+$). Rpyr values given refer to the center of each spot compared to the center of standard pyruvic acid.

[b] Not determined.

ness in vacuo at 40 C. A portion [e.g., as little as 1% of the sample (Table 3)] of the resulting preparation was then analyzed for amino acids by eluting with buffer at *p*H 3.15 on a Beckman 120-C amino acid analyzer. Only alanine was observed in the amino acid chromatograms of the final preparation (Table 3). This represents a 97% yield from the reduction step and a 78.6% overall yield. Thin-layer chromatography revealed that no alanine was present in the initial hydrolysate and in the ether extract, and no pyruvic acid or phenylhydrazone derivative was observed in the final reduction product. These results are shown in Fig. 1 and 2.

TABLE 2. *Release of pyruvic acid by hydrolysis with acid and base*

| Klebsiella strain | Conditions[a] | | |
|---|---|---|---|
| | A | B | C |
| A(1) | 8.0 ± 1.2 | 0 | 6.8 ± 1.0 |
| B(2) | 0.4 ± 0.05 | 0 | 0.4 ± 0.05 |
| C(3) | 3.4 ± 0.7 | 0 | 3.4 ± 0.7 |
| D(4) | 7.7 ± 1.2 | 0 | 8.2 ± 1.2 |
| E(5) | 5.5 ± 0.8 | 0 | 5.5 ± 0.8 |
| F(6) | 8.9 ± 1.3 | 0 | 8.0 ± 1.2 |
| 68 | 1.7 ± 0.2 | 0 | 2.4 ± 0.4 |
| G-SS-12 | 7.0 ± 1.0 | 0 | 7.0 ± 1.0 |
| 13884 | 7.5 ± 1.1 | 0 | 7.5 ± 1.1 |

[a] Values are expressed as grams of pyruvate released per 100 g of capular polysaccharide. (A) 0.01 N HCl at 100 C for 2.5 hr, (B) 0.2 N NaOH at room temperature for 2.5 hr, and (C) first with 0.2 N NaOH at room temperature for 2.5 hr followed by neutralization and hydrolysis with 1 N HCl at 100 C for 1.5 hr. Pyruvic acid released was measured by the Friedemann and Haugen procedure (4).

TABLE 3. *Identification and quantification of amino acid obtained by catalytic hydrogenation of 2,4-dinitrophenylhydrazone of klebsiella α-keto acids*

| α-Keto acid | Amino acid analogue | Retention time of known amino acid (min)[a] | Amt of unknown amino acid (μmoles)[b] |
|---|---|---|---|
| Glyoxylic acid | Glycine | 101 | <0.001 |
| Pyruvic acid | Alanine | 109.5 | 0.062 |
| β-Hydroxy-pyruvic acid | Serine | 66 | <0.001 |
| β-Thiopyruvic acid | Cysteine | 121.5 | <0.001 |
| α-Keto-*n*-butyric acid | α-Amino-*n*-butyric acid | 119 | <0.001 |

[a] Retention times of amino acid analogues of certain α-keto acids eluted at *p*H 3.15 on the Beckman 120-C Amino Acid Analyzer were as described previously (16).

[b] Amounts of each amino acid observed in chromatograms of 1% of total hydrazone reduction products obtained from a mixture of klebsiella polysaccharides and 2,4-dinitrophenylhydrazones.

## DISCUSSION

Appreciable amounts of an acid-labile, diethyl-ether-soluble α-keto acid was demonstrated to occur in all strains of klebsiella polysaccharides examined, with the exception of *K. ozaenae* serotype B(2) preparations which contained very little α-keto acid. These compounds could be detected with carbonyl group reagents such as semicarbazide and *p*-nitrophenylhydrazine on thin-layer

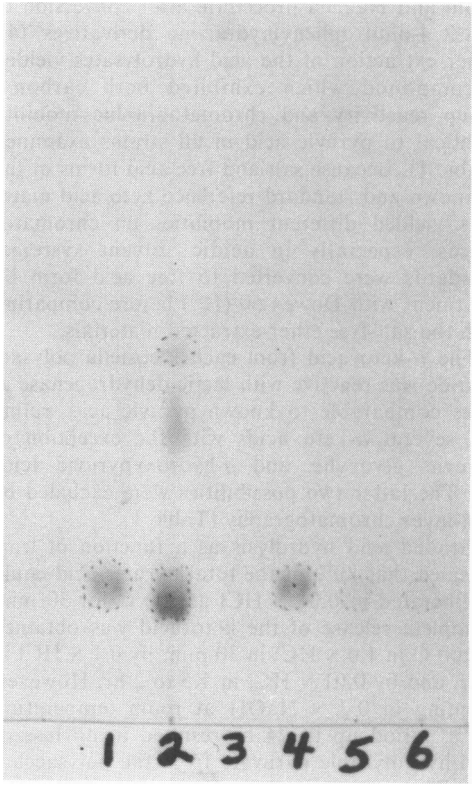


FIG. 1. *Thin-layer chromatogram showing standard alanine, spots 1 and 4; products from palladium-catalyzed hydrogenation of klebsiella α-keto acid 2,4-dinitrophenylhydrazone ether extract, 2; unreduced klebsiella α-keto acid 2,4-dinitrophenylhydrazone ether extract, 3; crude hydrolysate (0.01 N HCl, 90 min, 100 C) of klebsiella mixed polysaccharides before reaction with 2,4-dinitrophenylhydrazine, 5; and standard sodium pyruvate, 6. The solvent system was ethylacetate-pyridine-acetic acid-water (5:5:1:3), and the plate was sprayed with ninhydrin. Equal amounts of the preparation were spotted in each instance. The two fast-moving spots from 2 were present as yellow spots before spraying with ninhydrin, whereas control spots 5 and 6 did not stain blue and did not appear until several days after spraying.*

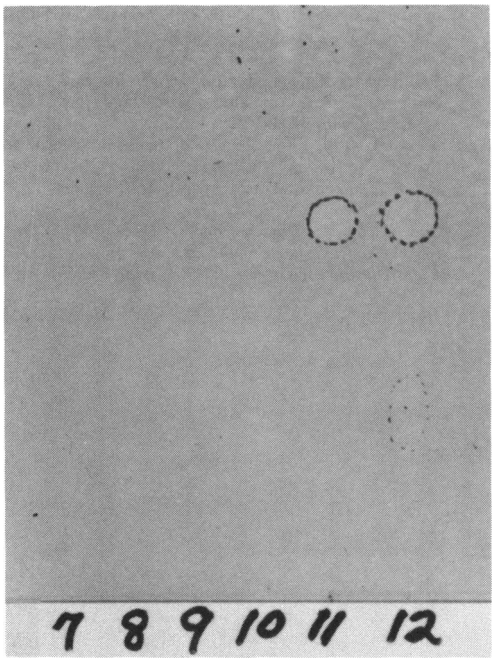


FIG. 2. *Thin-layer chromatogram of the same fractions as shown in Fig. 1. Spots 1 to 6 of Fig. 1 are identical to spots 7 to 12, respectively, of Fig. 2. The solvent system and amounts of the various fractions are the same as described in Fig. 1; 0.5% p-nitrophenylhydrazine-1 N NaOH spray was used to detect keto compounds.*

plates, or as 2,4-dinitrophenylhydrazones in the Friedemann and Haugen assay for $\alpha$-keto acids, and by reaction with lactic dehydrogenase and reduced nicotinamide adenine dinucleotide. Derivative formation, thin-layer chromatographic mobilities, and reactivity with lactic dehydrogenase indicated the compound to be pyruvic acid, and characterization as pyruvic acid was achieved by catalytic reduction to alanine of the 2,4-dinitrophenylhydrazone derivative. These results confirmed our previous observation of the occurrence of pyruvic acid in *K. rhinoscleromatis* K type 3- or type 3(C)-specific polysaccharide (18) and our preliminary report of the occurrence of pyruvic acid in the other klebsiella strains studied here (11). Since completion of this work, Heidelberger and co-workers have also reported the occurrence of pyruvic acid in several different klebsiellae, including K types 3, 9, 32, 47, and 52 (reference 7).

The definitive characterization of pyruvic acid by catalytic palladium hydrogenation of the 2,4-dinitrophenylhydrazone derivative to alanine in 97% yield and assay of about 0.03 $\mu$moles of this compound on an amino acid analyzer indicates the facility of this procedure and its application in working with small amounts of materials. Such a procedure should be useful where the occurrence of pyruvic acid has been noted in other bacterial polysaccharides (3, 7, 9, 10, 15, 17).

The observation reported here, that pyruvic acid release from klebsiella polysaccharides was effected by acid but not alkali, indicates ketosidic rather than ester linkages. Similar results were reported in studies of rhizobial polysaccharides (3) and were more recently suggested again in studies on the immunochemical relationships of certain capsular polysaccharides of klebsiella, pneumococci, and rhizobia (7). Ketal linkages have been shown to link pyruvic acid to polysaccharides produced by *X. campestris* NRRL B-1459 (14, 15), *C. insidiosum* (6), and more recently in the colanic acids produced by *Escherichia coli* S. 53, *Salmonella typhimurium* SL 1543, and *Aerobacter cloacae* NCTC 5920 (10, 17).

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